Int. J. Electrochem. Sci., 16 **(2021)** Article ID: 210913, doi: 10.20964/2021.09.12

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Electrochemical DNA Biosensor Based on thiolated-MWCNTs modified glassy carbon electrode for Determination of Protein and Hemoglobin in Urine at Exercise Training Levels

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Received: 29 April 2021/ *Accepted:* 21 June 2021 / *Published:* 10 August 2021

The synthesis of conjugated glycated hemoglobin (gHb)-DNA on thiolated-MWCNTs modified glassy carbon electrode (gHb-DNA/MWCNTs/GCE) and application as a sensor of nuclear matrix protein 22 (NMP22) and hemoglobin (protein inside red blood cells) in urine specimens at different levels of exercise training was studied. Surface analysis of functionalized MWCNTs/GCE using SEM analysis revealed that formation entangled f-MWCNT network can act as a porous conductive substrate for immobilization DNA. Electrochemical analyses were performed using DPV and amperometry. The electrochemical results showed the simultaneous determination of Hb and NMP22 with higher stability and sensitivity on gHb-DNA/MWCNTs/GCE toward f-MWCNTs/GCE,gHb-DNA/GCE. The amperometric analyses of gHb-DNA/MWCNTs/GCE showed that the linear range, limit of detection and the sensitivity for determination NMP22 were obtained 1 to 100µg/mL, 0.007ng/mL and 10.4146µA/µg mL^{-1}, respectively. The linear range, the limit of detection, and the sensitivity for determination of NMP22 were obtained 1 to 100µg/mL, 0.005ng/mL and 14.0865µA/µg mL^{-1}, respectively. The selectivity and practical ability of gHb-DNA/MWCNTs/GCE for determination of Hb and NMP22 were examined in presence of various interfering species and prepared clinical urine specimens. The results indicated a selective, reliable, and accurate response of gHb-DNA/MWCNTs/GCE as electrochemical Hb and NMP22 sensors.

Keywords: DNA Biosensors; Nuclear matrix protein 22; Hemoglobin, Urine; Electrochemical analysis

1. INTRODUCTION

Cellular metabolism in the human body creates many by-products that are often nitrogen-rich species such as urea, uric acid, and creatinine which must be cleared from the bloodstream [1, 2]. The color, odor and amount of urine can already indicate whether something is wrong [3]. Urine contains 95% of water, and an assortment of inorganic salts such as sodium, chlorides, phosphates, sulfates and

ammonia, and organic compounds such as urea, uric acid, proteins, hormones and metabolites [4, 5]. Analyses of the generated by-products in the urination process as the primary method can help to determine excreted water-soluble chemicals from the body [6, 7].Urine tests can help to diagnose diseases of the urinary system as well as diabetes, kidney, gastrointestinal diseases and liver disease [8, 9].

Studies have been shown that the techniques including microscopy [10], photoelectricity [11], biuret [12], photometry [13], microcolorimetry [14, 15], spectrophotometry [16], high-performance liquid chromatography/electrospray–mass spectrometry [17] and electrochemical methods [18, 19] are the urine test methods. However, the available methods have been shown to lack the desired accuracy, sensitivity, selectivity and convenience [20, 21]. These deficiencies have been caused to attempt to develop and improve the detection procedure [22-24]. A among these techniques, electrochemical methods are simple, low cost and fast, and the capability to modify the surface of the electrodes by various nanostructured materials promotes the sensing properties [25, 26].

Many electrochemical analyses of urine samples have been conducted on the gold, platinum and silver electrodes [27-31]. As consequence, identify and synthesis of low cost and stable materials for modification of urine electrochemical test electrodes is necessary. Therefore, this study was focused on the synthesis and application of gHb-DNA/MWCNTs modified GCE as a sensor of NMP22 and hemoglobin as protein inside red blood cells in urine specimens at exercise training levels.

2. EXPERIMENTAL

Before modification the GCE, GCE was carefully polished on a polishing cloth with 0.05 μm alumina powder (99%, Liaoning Hongtong Metallurgical Refractory Co., Ltd., China) to a mirror finish, and then rinsed with deionized (DI) water. After that, GCE was ultrasonically washed in mixture of ethanol and water for 10 minutes. For functionalization of MWCNTs [32], a certain amount of MWCNTs (99%, Dongguan Sat Nano Technology Material Co., Ltd., China) were treated with a mixture of HNO_3 (65%, Shijiazhuang Chemical Tech Co., Ltd., China) and H_2SO_4 (98%, Sigma-Aldrich) solution in a volume ration of 3:1 at 65°C for 120 minutes. Functionalization caused to create open edge sites and covalent bonding between amine groups of the hardener and carboxylic groups can form a network with more charge transfer sites and strong connection for immobilization biomolecules and bifunctional hardener molecule [33]. The f-MWCNTs were rinsed with DI water and dried at room temperatures. Then, f-MWCNTs were ultrasonically dispersed in dimethylformamide (DMF, 99.8 %, Sigma-Aldrich) for 10 minutes to reach the homogenous suspension.

In order to modification the GCE with f-MWCNTs [34], an appropriate volume of the f-MWCNTs suspension was drop-casted on the electrode surface. The GCE was transferred to an oven at 55°C for 15 minutes. For prepared thiolated MWCNTs on GCE [35], after cooling, the f-MWCNTs/GCE was immersed in a mixture of 10 g thiourea (99.9 %, Sigma-Aldrich), 25 mL hydrobromic acid (47%, Merck) and 50 mL glacial acetic acid (100%, Merck) was at 4°C for 24 hours. Subsequently, the thiolated-MWCNTs/GCE was rinsed with 0.1 M PBS and stored in a refrigerator at 4°C.

Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, ≥97%, Sigma-Aldrich) was used for conjugating and formation link between glycated hemoglobin (gHb, Merck) and DNA as following procedure [36]: the mixture of 10 μM of DNA (5′-AAAATAAAAACGCGCGGAACCCCCGCGC-3′, Bioneer, South Korea), gHb, Tris–EDTA baffer (Sigma-Aldrich) and dithiothreitol (DTT, ≥99.0%, Merck) were dissolved in 10μM Tris–EDTA in equal volume ratio. Next, ethyl acetate (99.8 %, Sigma-Aldrich) the solution was used to remove consumed DTT from solution. Then, 10mM Sulfo-SMCC was added to the mixture solution in a volume ratio of 1:4. The obtained solution was stored in refrigerator at 4°C for 120 minutes. After then, 10 μM of hemoglobin solution was added to the obtained solution in equal volume ratio, and the final solution was stored again in a refrigerator at 4 °C for 3 hours. Thereby, amine group of gHb was conjugated with the thiol on DNA-Sulfo SMCC [36]. Subsequently, the prepared mixture of conjugated gHb -DNA was stored in the refrigerator at 4°C.

For immobilization of conjugated gHb-DNA on the thiolated-MWCNTs/GCE, the electrode was immersed at a mixture of 0.1 M phosphate buffer solution (PBS) pH 7 and conjugated gHb-DNA solution in equal volume ratio for 60 minutes. The obtained gHb-DNA/MWCNTs/GCE was rinsed with 0.1 M PBS and stored in a refrigerator at 4°C.

The electrochemical measurements using differential pulse voltammetry (DPV) and amperometry were conducted on Auto lab potentiostat/galvanostat (PGSTAT30, Eco Chemie Autolab, Utrecht, The Netherlands) with three-electrode cell, which contained prepared electrode as working electrode, Ag/AgCl as a reference electrode and Pt plate as a counter electrode. The electrolyte for electrochemical studies was performed in 2mM $K_4[Fe(CN)_6]$ (≥99.95%, Sigma-Aldrich) and 0.1 M PBS pH 7 which prepared from 0.1M NaH_2PO_4 (97%, Xinxiang Huaxing Chemical Co., Ltd., China) and 0.1 M Na_2HPO_4 (99%, Shanghai Ruizheng Chemical Technology Co., Ltd., China).

Urine specimens at exercise training levels were collected and immediately stored in a refrigerator. 10 mL of samples were centrifuged at 1000 rpm for 15 minutes and obtained supernatant was filtered (0.45 μm pore size, MF-Millipore™ Membrane). Next, the solution was transferred into a volumetric flask (25 mL) and used 0.1 M PBS pH 7.0 to dilute to the mark. The diluted specimens were spiked with different amounts of hemoglobin. The samples were analyzed by amperometric technique and urine dipstick test (UDT).

The scanning electron microscopy (SEM, FEI Company Hillsboro, OR, USA) was used for study on the surface morphology of f-MWCNTs/GCE.

3. RESULTS AND DISCUSSION

Figure 1 shows the SEM image of the surface of bare GCE and f-MWCNTs/GCE. The interwoven is observed with a cobweb-like fabric that largely consists of MWCNTs. As observed, MWCNTs inhomogeneously are covered the GCE surface which is probably because of the surface tension gradient related to the droplet interface [37]. Moreover, this inhomogeneity can be associated with the coagulation of the f-MWCNTs during heat treatment [38], and non-uniform adhesion of f-MWCNTs on GCE [39]. Thus, the entangled f-MWCNT network not only can act as conductive

substrate but also behave as a probe, which would immobilize DNA strand and hybridize with the analyte DNA [40]. The average diameter and length of f-MWCNTs are 80nm and 4µm, respectively.

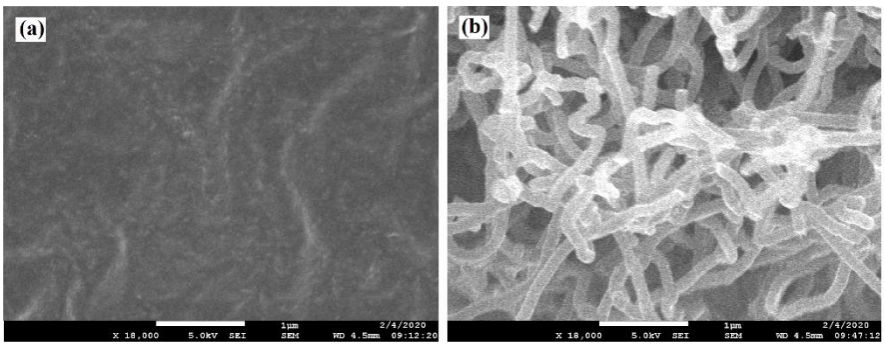


Figure 1. SEM image of Surface of (a) bare GCE and (b) f-MWCNTs/GCE

Figure 2 shows the DPV curve of GCE, f-MWCNTs/GCE,gHb-DNA/GCE, and gHb-DNA/MWCNTs/GCE at a scan rate of 20 mV/s in 0.1 M PBS pH 7 containing 1.5 µg/mL Hb. As observed from Figures 2a and 2b, there is a broad shoulder at 0.46 V for f-MWCNTs/GCE, and no significant peaks are obtained for GCE and in presence of Hb. However, in Figures 2c and 2d, the peaks are observed at 0.30 V and 0.23 V with current 10 and 13.7 µA as oxidation peaks of Hb for gHb-DNA/GCE and gHb-DNA/MWCNTs/GCE, respectively. The difference between the potential and current values on gHb-DNA/GCE and gHb-DNA/MWCNTs/GCE related to the presence of high conductive MWCNTs, which enhance the high conductivity and signal of electrochemical reaction. In addition, the good nanostructured entangled MWCNTs network improves fast electron transfer in the electrochemical reaction, and consequently, decreases the potential value [41, 42]. The high porosity of f-MWCNTs on GCE enhance the density of nanostructured active sites and effective surface area on modified electrode [42].

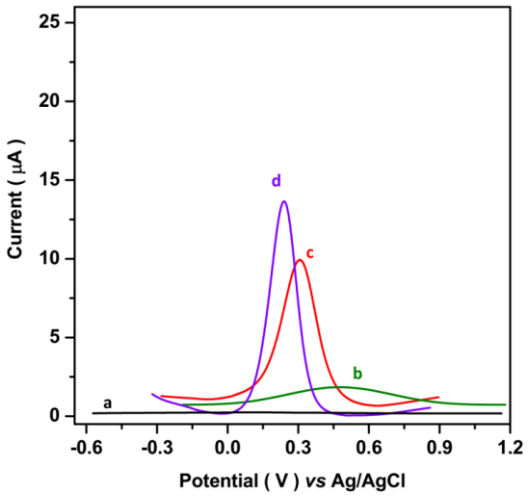


Figure 2. DPV curves of (a) GCE, (b) f-MWCNTs/GCE, (c) gHb-DNA/GCE and (d) gHb-DNA/MWCNTs/GCE in 0.1 M PBS pH 7 containing 1.5µg/mL Hb at scan rate of 20mV/s

Figure 3 shows the repeat of these studies for prepared electrodes at a scan rate of 20mV/s in 0.1 M PBS pH 7 containing 1.5µg/mL NMP22.

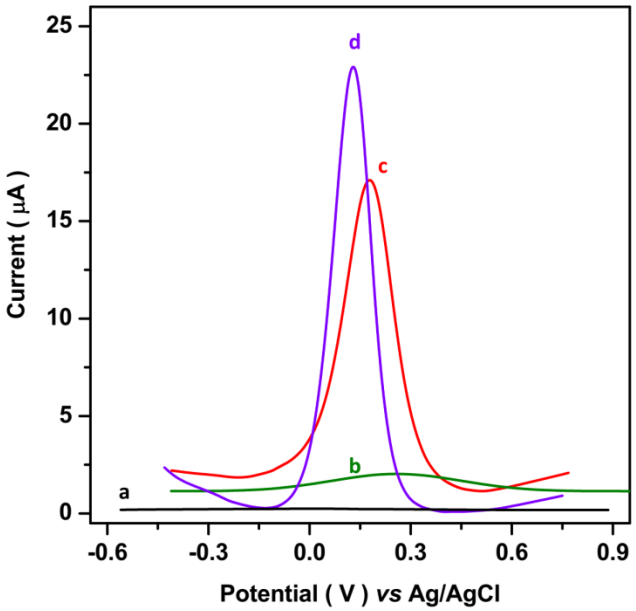


Figure 3. DPV curves of (a) GCE, (b) f-MWCNTs/GCE, (c) gHb-DNA/GCE and (d) gHb-DNA/MWCNTs/GCE in 0.1 M PBS pH 7 containing 1.5µg/mL NMP22 at scan rate of 20mV/s

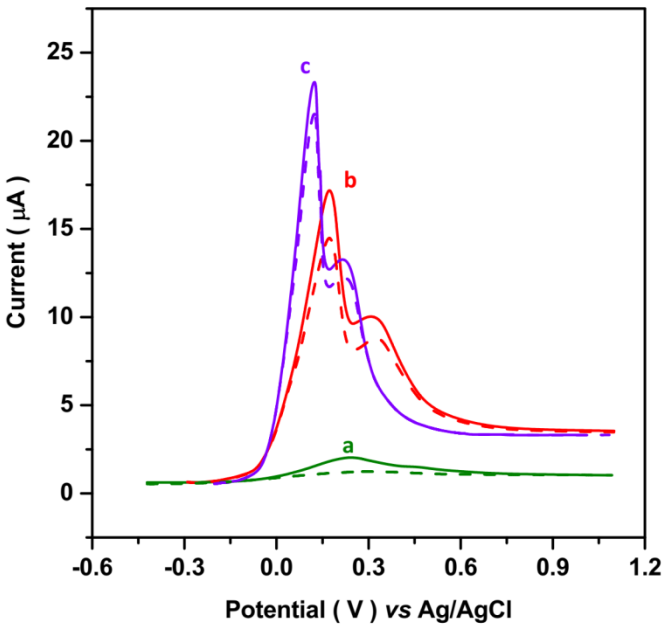


Figure 4. The second (solid line) and 50th (dash line) scans of DPV curves of (a) f-MWCNTs/GCE, (b) gHb-DNA/GCE and (c) gHb-DNA/MWCNTs/GCE at scan rate of 20 mV/s in 0.1 M PBS pH 7 containing 1.5 µg/mL Hb and NMP22.

From Figure 3, no obvious peaks are observed for GCE and f-MWCNTs/GCE, and significant oxidation peaks for NMP22 are observed at 0.17 V and 0.12 V with current 17 and 23 µA on gHb-

DNA/GCE and gHb-DNA/MWCNTs/GCE, respectively. These DPV curves also show the smaller potential and higher oxidation current are obtained on gHb-DNA/MWCNTs/GCE. The difference between the anodic potentials of Hb and NMP22 on gHb-DNA/GCE and gHb-DNA/MWCNTs/GCE indicate to the capability of prepared electrode to the simultaneous determination of both analytes through DPV analysis.

In order to study the stability of electrochemical responses of f-MWCNTs/GCE, gHb-DNA/GCE and gHb-DNA/MWCNTs/GCE, the successive DPV scans of prepared electrodes in PBS pH 7 containing 1.5 µg/mL Hb and 1.5 µg/mL NMP22 at scan rate 20 mV/s. Figure 4 shows that the weak peak current of f-MWCNTs/GCE is diminished after continues 50 scans. The second scans of gHb-DNA/GCE and gHb-DNA/MWCNTs/GCE show the simultaneously separated peaks for Hb and NMP22. The different between oxidation current of the second and 50^{th} DPVs indicates the decrease of Hb oxidation currents after 50 scans for gHb-DNA/GCE and gHb-DNA/MWCNTs/GCE are about ~18% and 6%, respectively. Moreover, the decreases of NMP22 oxidation currents after 50 scans are obtained about ~14% and 9% for gHb-DNA/GCE and gHb-DNA/MWCNTs/GCE, respectively. Accordingly, the higher stability responses are obtained on gHb-DNA/MWCNTs/GCE because of the synergetic effect of conjugated gHb-DNA on the thiolated-MWCNTs/GCE. These results show the important role of f-MWCNTs on immobilization of conjugated gHb-DNA on GCE surface [43]. Studies have been indicated that covalent attachment plays a more important role in CNT-based DNA electrochemical sensors [44]. The surface of MWCNT are activated by exposing it to acidic media to develop −COOH, –OH, C=O bonds and the probe DNA sequence is amine-modified at 5' end to covalently bind with the thiolated-MWCNTs, and enhances the bioactivity of the MWCNTs [43], and decrease the electron transfer resistance of DNA [44]. Therefore, gHb-DNA/MWCNTs/GCE was selected for the further electrochemical study of Hb and NMP22 due to its lower potential, high sensitivity, and stable response.

Figure 5a shows the amperometric responses ofgHb-DNA/MWCNTs/GCE to successive additions of 1µg/mL Hb in 0.1 M PBSpH 7.0 at a potential 0.23V. As seen, the amperometric current is increased with increasing the Hb concentration. Figure 5b reveals the calibration plot of electrocatalytic current vs. Hb concentration, indicating the linear relationship between oxidation current and Hb concentration. Moreover, the calibration graph starts to deviate from linear behavior for Hb concentration more than 100 µg/mL. It can be related to a typical limitation in biosensors with nanostructured outer membranes, which facilitates the conversion of the enzyme and reach a steady state, and this may lead to some accumulation of the substrate at the inner membrane boundary [45]. This undesirable factor can saturate the active site on the biosensor. The limit of detection and the sensitivity for determination Hb are obtained at 0.007ng/mL and 10.4146µA/µg mL^{-1}, respectively.

The amperometric response of gHb-DNA/MWCNTs/GCE and its calibration plot for successive addition of 1µg/mL NMP22 in 0.1 M PBSpH 7.0 at potential 0.12 V also are depicted in Figures 6a and 6b, respectively. The linear range, limit of detection and the sensitivity for determination NMP22 are obtained 1 to 100µg/mL, 0.005ng/mL and 14.0865µA/µgmL^{-1}, respectively.

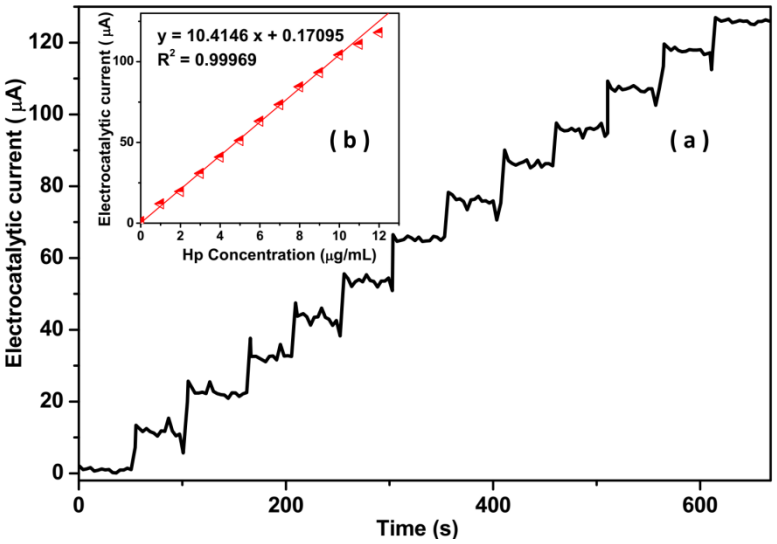
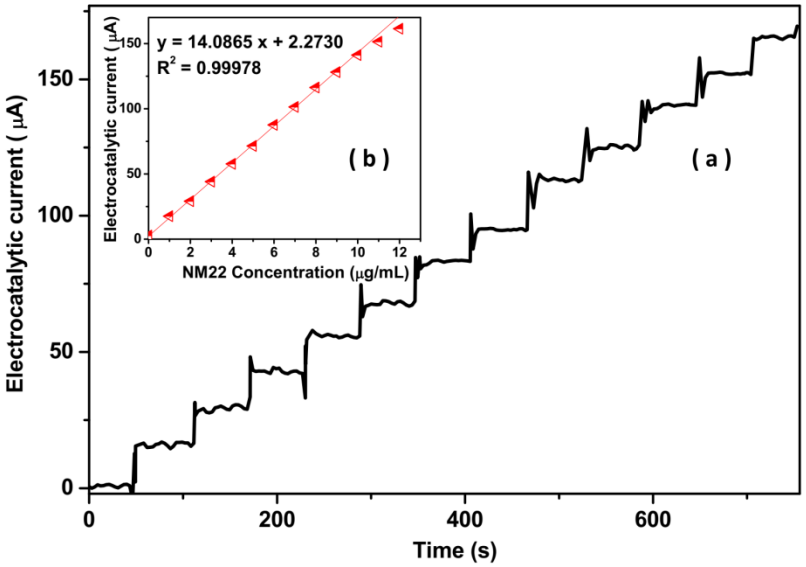


Figure 5. (a) Amperometric responses of gHb-DNA/MWCNTs/GCE to successive additions of 1μg/mL Hb in 0.1M PBS pH 7.0 at potential 0.23 V; (b) the calibration plot.



Figures 6. (a) Amperometric responses of gHb-DNA/MWCNTs/GCE to successive additions of 1μg/mL NMP22 in 0.1 M PBS pH 7.0 at potential 0.12V; (b) the calibration plot

The analytical sensing properties of the gHb-DNA/MWCNTs/GCE are compared with other reported Hb and NMP22 sensors. The comparison from Table 1 reveals that the obtained analytical results of gHb-DNA/MWCNTs/GCE are acceptable and the linear range is higher than other technique and electrochemical sensors because of MWCNT's high stability, fast response and easy operation. This CNT-based electrochemical biosensor can act as a biocatalytic and affinity sensor [44]. In addition, many studies have been conducted using expensive noble metals such as Au and Pt [27-31],

however, application of the carbon nanotube in biosensor architecture for immobilization of DNA can be economical.

Table 1. Comparison between the analytical sensing properties of the gHb-DNA/MWCNTs/GCE and other reported Hb and NMP22 sensors.

Electrodes	Analyte	Technique	Detection limit (ng/mL)	Linear range (µg/mL)	Sensitivity (µA/mg mL^{-1})	Ref.
gHb-DNA/MWCNTs/GCE	Hb	amperometry	0.007	1-100	10414.6	This work
gHb-DNA/MWCNTs/GCE	NMP22	amperometry	0.005	1-100	14086.5	This work
rGO-tetraethylenepentamine /AuNPs-PtNPs-MOFs	NMP22	DPV	0.0017	5×10^{-6}-0.02	8533600	[27]
horse radish/Ab NMP22/Fe_3O_4/ Au/Co-phthalocyanine/Au colloid - modified gold electrode	NMP22	amperometry	0.5	1.2×10^{-3} – 0.2	-	[28]
Au@Pd/Ag NPs	NMP22	SWV[a]	0.0033	10^{-1}-18 $\times10^{-3}$	5.886	[29]
g-C3N4/Au NPs	NMP22	CV[b]	0.010	5×10^{-5} - 2×10^{-3}	-	[30]
rGO-NH/NH_2-silicoaluminophosphates -34-Pd/Co	NMP22	amperometry	0.00033	1×10^{-3} - 2 $\times10^{-3}$	0.434	[46]
SiO2–Au/GCE	Hb	DPV	204.46	5-5×10^{2}	83.14106	[31]
Methylene blue/perfluorosulfonated ionomer /Nafion/microcylinder	Hb	Amperometry	13	2.35×10^{-2} - 0.235	-	[47]
Iodide/Ag	Hb	DPV	1950	2.35×10^{-3}– 2.35×10^{-2}	21490	[48]
Cu-based metal organic frameworks-macroporous carbon	Hb	Amperometry	22.75	6.5×10^{-2}- 1.56×10^{-2}	1.28	[49]
Fc[CO-Glu-Cys-Gly-OH]	Hb	DPV	30	0.1–1000	1.09	[50]
Nile Blue/GCE	Hb	Amperometry	-	50 -7000	-	[51]
Acid chrome blue K/GCE	Hb	Amperometry	64	0.325–64	0.037	[52]

[a]Squarewave voltammetry, [b] Cyclic voltammetry

The analysis of clinical urine specimens using electrochemical sensor detection strategy has the greatest challenge due to frequently and efficiency in recognize medication. For study the selective performance of gHb-DNA/MWCNTs/GCE as Hb sensor, nitrite, glucose, serotonin, dopamine, ascorbic acid as the species in urine were selected for study of interference effect because of their similar oxidation potentials at most solid electrodes, and formation of overlapping signals [53]. The electrochemical interference effect of Ca^{2+}, K^{+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Pb^{+2}, Fe^{3+}, Ni^{2+}, Al^{3+} and Hg^{2+} ions on determination Hb and NMP22 were investigated. Table 2 presents the amperometric response of gHb-DNA/MWCNTs/GCE in 0.1 M PBS pH 7 at 0.23V for successive addition of 1µg/mL Hb and 20 mg/mL of interferents. As seen, the proposed electrode illustrates a significant response to additions of Hb solution, and additions of other interferents do not show any obvious response for the additions of interferents at 0.23V. Table 2 also presents a similar measurement for investigating the interference

effect on NMP22 determination at 0.12V. The results from Table 2 show the obvious signal for NMP22 and the presented species in Table 3 did not present any interference effect on determination NMP22.

Table 2. The amperometric responses of gHb-DNA/MWCNTs/GCE in 0.1 M PBS pH 7 at 0.23V and 0.12 V for successive addition of 1µg/mL Hb and NMP22, and 20µg/mL of interferents.

substance	Added (µg/mL)	Electrocatalytic current response (µA) at 0.23 V	RSD (%)	Electrocatalytic current response (µA) at 0.12 V	RSD (%)
Hb	1	10.514	±0.211	0.391	±0.003
NMP22	1	0.432	±0.010	14.086	±0.197
Nitrite	20	0.270	±0.011	0.124	±0.015
Glucose	20	0.361	±0.007	0.132	±0.005
Serotonin	20	0.165	±0.007	0.152	±0.008
Dopamine	20	0.244	±0.007	0.115	±0.002
Ascorbic acid	20	0.215	±0.003	0.151	±0.003
Ca^{2+}	20	0.110	±0.002	0.095	±0.002
Pb^{2+}	20	0.076	±0.03	0.108	±0.003
Ni^{2+}	20	0.119	±0.002	0.117	±0.004
Fe^{3+}	20	0.094	±0.002	0.089	±0.002
Cu^{2+}	20	0.080	±0.003	0.078	±0.005
Mg^{2+}	20	0.099	±0.005	0.120	±0.002
K^{+}	20	0.077	±0.004	0.119	±0.004
Zn^{2+}	20	0.071	±0.007	0.117	±0.005
Al^{3+}	20	0.067	±0.002	0.108	±0.003
Hg^{2+}	20	0.110	±0.002	0.091	±0.003

Table 3. The analytical results of determination of Hb and NMP22 prepared clinical urine specimens using amperometric and UDT techniques.

Amperometry						UDT	
Original found (µg/mL)		Added (µg/mL)	Measured (µg/mL)	RSD (%)	Recovery (%)	Content in sample (µg/mL)	RSD (%)
Hb	5.1	10.0	14.9	1.97	98.00	5.4	2.02
		20.0	24.8	2.73	98.50		
		30.0	35.0	3.11	99.66		
		40.0	44.8	4.02	99.25		
NMP22	2.2	10.0	11.8	1.89	96.00	2.3	2.11
		20.0	22.1	2.44	99.50		
		30.0	31.9	3.24	99.00		
		40.0	42.0	4.13	99.50		

The validity, accuracy and practical ability of gHb-DNA/MWCNTs/GCE to determination of Hb and NMP22 were examined in prepared clinical urine specimens. Table 3 shows the results of the

determination of the two species in real samples by amperometric and UDT measurements which indicated to acceptable agreements between the amperometric and UDT results, and good agreement with the SI units for clinical laboratory data [54]. Satisfactory recovery and mean relative standard deviation (RSD) are given in Table 3. The results demonstrates the obtained recovery values more than 98.00% and 96.00% for determination Hb and NMP22, and RSD values less than 4.02% and 4.13% for determination of Hb and NMP22, respectively. These results show the reliable and accurate response of gHb-DNA/MWCNTs/GCE as electrochemical Hb and NMP22 sensors.

4. CONCOUSION

This study was carried out on the synthesis of gHb-DNA/MWCNTs/GCE and applicationas sensor of NMP22 and hemoglobin in urine specimens at exercise training levels. Morphology analysis of f-MWCNTs/GCE exhibited the porous formation of f-MWCNT network on GCE. The electrochemical results indicated that gHb-DNA/MWCNTs/GCE shows the simultaneous determination of Hb and NMP22 with higher stability and sensitivity signal than f-MWCNTs/GCE, gHb-DNA/GCE. The amperometric analyses of gHb-DNA/MWCNTs/GCE showed that the linear range, limit of detection, and the sensitivity for determination of NMP22 were obtained 1 to 100μg/mL, 0.007ng/mL, and 10.4146 μA/μg mL^{-1}, respectively. The linear range, limit of detection and the sensitivity for determination of NMP22 were obtained 1 to 100μg/mL, 0.005ng/mL and 14.0865μA/μg mL^{-1}, respectively. The selectivity and practical ability of gHb-DNA/MWCNTs/GCE to the determination of Hb and NMP22 were investigated in presence of various interfering species and prepared clinical urine specimens. The results showed a selective, reliable and accurate response of gHb-DNA/MWCNTs/GCE as electrochemical Hb and NMP22 sensors.

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